The plasma 5′-AMP acts as a potential upstream regulator of hyperglycemia in type 2 diabetic mice

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Zhang Y, Wang Z, Zhao Y, Zhao M, Wang S, Hua Z, Zhang J. The plasma 5′-AMP acts as a potential upstream regulator of hyperglycemia in type 2 diabetic mice. Am J Physiol Endocrinol Metab 302:E325–E333, 2012. First published November 8, 2011; doi:10.1152/ajpendo.00424.2011.—Increased plasma free fatty acid (FFA) level is a hallmark of type 2 diabetes. However, the underlying molecular basis for FFA-caused hyperglycemia remains unclear. Here we identified plasma 5′-adenosine monophosphate (pAMP) markedly elevated in the plasma of type 2 diabetic mice. High levels of FFAs induced damage in vein endothelial cells and contributed to an increase in pAMP. Administration of synthetic 5′-AMP caused hyperglycemia and impaired insulin action in lean wild-type mice. 5′-AMP elevated blood glucose in mice deficient in adenosine receptors with equal efficiency as wild-type mice. The function of pAMP was initiated by the elevation of cellular adenosine levels, directly stimulating G-6-Pase enzyme activity, attenuating insulin-dependent GLUT4 translocation in skeletal muscle, and displaying a rapid and steep increase in blood glucose and a decrease in hepatic glycogen level. It was followed by an increase in the gene expression of hepatic Foxo1 and its targeting gene Pepck and G6Pase, which was similar to diabetic phenotype in db/db mice. Our results suggest that pAMP is a potential upstream regulator of hyperglycemia in type 2 diabetes.

adipose 5′-monophosphate; free fatty acids; hyperglycemia; glucose-6-phosphatase; glucose transporter 4

Type 2 diabetes mellitus, a common metabolic disease increasing at an alarming rate worldwide, is defined as chronic hyperglycemia resulting from multiple genetic and environmental factors (25, 37). The leptin-receptor-deficient (db/db) mouse has been identified as a monogenic model for diabetes resulting from impaired GLUT4 translocation in response to insulin (11). Defects in skeletal muscle GLUT4 translocation have been observed consistently in various animal models of insulin resistance and type 2 diabetes (26, 38, 60). Overexpression of GLUT4 in muscle increased 2.5-fold the in vivo insulin-stimulated glucose uptake in fast-twitch skeletal muscles, and these mice exhibited increased whole body glucose utilization (53). High levels of plasma FFAs also caused hepatic insulin resistance by inhibiting insulin-mediated suppression of glycogenolysis (46) and interfered with the suppression of endogenous glucose production, resulting in an excessive hepatic glucose output, which is an important factor in the development of hyperglycemia in diabetic mice and subjects (54, 56).

The underlying molecular basis for increased FFAs causing impaired insulin action and glucose metabolism in type 2 diabetes remains unclear. Here, we identified that plasma 5′-adenosine monophosphate (pAMP) was abnormally elevated in the db/db mice. Elevated pAMP caused hyperglycemia and impaired insulin action through an increase in levels of cellular adenosine. Our results suggest that abnormally high levels of FFAs induced an increase in pAMP level, which is a potential upstream regulator of hyperglycemia in type 2 diabetes.

Materials and Methods

Experimental animals. Male C57BL/6 and C57BL/Ks db/db mice, 6–8 wk old, were used in this study. An identical response to 5′-AMP injection was also observed in female mice. Animals were maintained on 12:12-h light-dark cycles, with the light on at 0700 and off at 1900 and with free access to regular food and water. To create diet-induced type 2 diabetes animal models, 4-wk-old male mice were fed a high-fat diet (60% of calories were in the form of fat, 20% were in the form of protein, and 20% were in the form of carbohydrates) for 18 wk. All procedures were approved by Animal Care and Use Committee at Nanjing University of Science and Technology (NJUST).

Tissue and blood sampling. Mice were euthanized by cervical dislocation. Blood was collected from the carotid arteries in anticoagulant tubes containing a stop solution [0.2 mmol/l dipyridamole, 5 mmol/l erythro-9-(2-hydroxy-3-nonyl)-adenine, and 4.2 mmol/l EDTA], and then livers were removed and freeze-clamped in liquid nitrogen within 5 s. Blood samples were immediately centrifuged at 5,000 g for 5 min at 20°C. The plasma samples obtained were then stored on ice and immediately used in the experiments.

Incubation of human umbilical vein endothelial cells with FFAs. Human umbilical vein endothelial cells (HUVECs) were propagated in M199 and DMEM with 20% fetal bovine serum (FBS), 100 U·100 μg·1·mL−1 penicillin-streptomycin, 2 mM l-glutamine, 5 μg/ml endothelial cell growth supplement, and 17.85 IU/ml heparin at 37°C.
under 5% CO₂-95% air. FFA stock was a mixture of oleic acid and palmitic acid with a molar ratio of 2:1 and was prepared as described previously (58). The cells were incubated in a culture medium containing FBS, of which (FFA groups) albumin-conjugated long-chain FFAs were added in some cells (final concentrations 1 or 2 mM FFA); in some other cells (control groups), lipid-free BSA was added. After 24 or 48 h, cell necrosis was determined by exposing the HUVECs to a solution containing the DNA-binding dye propidium iodide (PI). PI enters those cells with a damaged membrane, staining the DNA red. Cells were then observed under a fluorescence microscope.

**HPLC analysis of uric acid, nucleotides, and adenosine.** Uric acid (UA), nucleotides (ATP, ADP, and 5'-AMP), and adenosine were extracted from frozen samples using 0.4 N perchorlic acid and analyzed by HPLC, as described previously (5, 27). Extracts were separated and quantified using reverse-phase HPLC (Waters 1525 system; Millipore, Bedford, MA) on a Partisil bonded phase C18 (reverse phase) cartridge column. Pure nucleotide and purine derivative (Sigma) solutions were used to identify the peaks and obtain the calibration curves. Characteristic peak spectra and retention times were used to identify nucleotides. Quantitation was based on the area under the peaks.

**Treatment with 5'-AMP, other nucleotides, insulin tolerance test, and pyruvate tolerance test.** The indicated doses of 5'-AMP, ATP, adenosine, inosine, cytidine monophosphate (CMP), inosine 5'-monophosphate (IMP), and UA (Sigma) were solvated in PBS and administered to mice by intraperitoneal injection. The same volume of saline (PBS) was injected as a control. All injections were given at zeitgeber time 2. For the insulin tolerance test, mice were fasted for 12 h and injected intraperitoneally with regular insulin (100 U/ml), resulting in a final concentration of 0.125 U/kg body wt or a mixture of 5'-AMP and insulin. For the measurement of pyruvate tolerance test (PTT), mice were fasted overnight (12 h) and then injected intraperitoneally with 2 g pyruvate/kg body wt or a mixture of 5'-AMP and pyruvate. After injection, mice were maintained for the desired length of time; blood glucose was determined before and after injection using a One Touch Blood Glucose Meter (AW063-436-01A; LifeScan), with 3 μl of whole blood obtained by tail bleed. Additionally, treated mice were euthanized and plasma and tissue samples collected for analysis. Hepatic glycogen was determined with a glycogen assay kit (KeyGEN, Nanjing, China).

**Immunofluorescence analysis for GLUT4.** Mice were either fasted overnight and left untreated or allowed to eat ad libitum and then given an intraperitoneal injection of glucose (1 g/kg) and insulin (8 U/kg) or a mixture of 5'-AMP, glucose, and insulin. Thirty minutes after injection, mice were anesthetized, and skeletal muscle was immediately removed and frozen in liquid nitrogen for frozen sectioning. After blocking for 30 min in PBS solution containing 5% donkey serum (Sigma) and 5% bovine serum albumin (American Bioanalytical, 4-μm frozen sections were stained with the primary anti-GLUT4 for 30 min at 37°C, rinsed three times with PBS, and stained for 30 min with the FITC-conjugated secondary antibody. The stained frozen sections were observed with fluorescence microscopy (Eclipse 800; Nikon, Tokyo, Japan).

**Western blotting analysis.** Fresh tissues were homogenized, and protein was extracted with Extraction Reagents (KeyGEN) according to the manufacturer’s instructions. The extracted protein was used for Western blot. AMP-activated protein kinase (AMPK) and phospho-AMPK antibodies were purchased from Cell Signaling Technology. Proteins were separated by electrophoresis through 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. Western blot analyses were performed as general protocol.

**Measurement of oxygen consumption.** Oxygen consumption was performed in metabolic chambers at a controlled ambient temperature (23 ± 2°C). A constant air flow (0.5 l/min) was drawn through the chamber and monitored using a metabolic monitor (AccuScan Instruments). After 30 min were allowed for adaptation to the metabolic chamber, mice were injected with 5'-AMP or saline, and Vo₂ was determined at 15-s intervals for a 24-h period. Mice had free access to water and food during the measurement period.

**Quantitative real-time RT-PCR analysis.** Mice were injected with 5'-AMP or saline. At 1 h after injection, total RNA was extracted from the liver sample using Trizol Reagent, and the remaining RNA was completely removed by RNase-free DNase treatment. Total RNA (50 ng) was reverse-transcribed with poly(dT)12-18 as a first-strand primer according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed, and the results were analyzed using an ABI 7300 Detection System in combination with SYBR green dye. The primer sequences are shown in Table 1. Relative gene expression compared with β-actin expression was calculated by the comparative threshold cycle method.

**Measurement of glucose-6-phosphatase activity enzyme activity and insulin-binding experiment.** Mouse livers frozen by liquid nitrogen were homogenized in homogenization buffer (0.25 M sucrose, 10 mM HEPES, pH 7.4) as described previously (29, 43). The suspension was incubated with 10 mM Tris-HCl (pH 7.3) and 20 mM glucose-6-phosphate (G6P) in a final volume of 200 μl at 37°C for 10 min. The reaction was stopped by adding 660 μl of 3.5% TCA. After centrifugation at 10,000 × g for 15 min at 4°C, 20 μl of supernatant was used to determine the amount of inorganic phosphate liberated from G6P, 50 μl of 2.5% ammonium molybdate and 50 μl of 10% ascorbic acid was added in a total volume of 500 μl for 5 min of boiling, and the optical density was measured at 800 nm (39). For adenosine-stimulating experiments, the homogenates were incubated with different concentrations of adenosine for 15 min and then used for determinations. The results were expressed as units (U) of enzymatic activity of 1 μmol of substrate hydrolyzed per minute per gram of wet liver. Insulin binding test was performed by measuring [125I]-insulin from isolated red blood cells, as described previously (34).

**Statistical analysis.** Data are presented as means ± SE. Statistical analysis was performed with Student’s t-test. Significance was defined by P < 0.05.

**RESULTS**

The plasma 5'-AMP and UA levels were abnormally elevated in type 2 diabetic mice. To identify possible changes in extracellular nucleotides and their possible role in influencing blood glucose homeostasis, we looked for differences in cir-

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**Table 1. Primer sequences for real-time RT-PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
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<tbody>
<tr>
<td>GLUT4</td>
<td>5'-AGCTGGATATTGCGTCAACGGC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GTGCCTCTGCTATGATCCTTT-3'</td>
</tr>
<tr>
<td>GLUT2</td>
<td>5'-GTGTTAAAACACGGTGGAAGACC-3'</td>
</tr>
<tr>
<td>Forward</td>
<td>5'-CTGGGTTCTGGAATTTTGCTA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TGTAAAAACACGGTGGAAGACC-3'</td>
</tr>
<tr>
<td>GLUT-6</td>
<td>5'-GACTCCCAAGAGCTGCTT-3'</td>
</tr>
<tr>
<td>Pax</td>
<td>5'-GGGCGTTTGGACAACAGAAT-3'</td>
</tr>
<tr>
<td>PEPCK</td>
<td>5'-ACTTTTGTGTCGCTGGATACCT-3'</td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GCCATGGGGCAGGTTATTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CGCAAGATGCGGAGCGAAGC-3'</td>
</tr>
<tr>
<td>FoxO1</td>
<td>5'-TCAAGAAATAGGGGCGAGCC-3'</td>
</tr>
<tr>
<td>Forward</td>
<td>5'-TGGTCATGAGCGACGCTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TGATGATGGCTGCTTCTCAGG-3'</td>
</tr>
</tbody>
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GLUT2 and -4, glucose transporter 2 and 4, respectively; G-6-Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; FoxO1, forkhead box O1.
cyclic nucleotides and their metabolites between \( db/db \) diabetic and lean wild-type mice. Plasma extracts obtained from \( db/db \) and lean wild-type mice were fractionated by reverse HPLC. Our analysis indicated that the levels of pAMP in \( db/db \) mice were elevated compared with those in lean mice (Fig. 1A). HPLC analysis also showed that the levels of UA were elevated in the plasma of \( db/db \) mice, indicating a significant difference between \( db/db \) and wild-type lean mice (Fig. 1A, right). The high-fat diet-fed mouse model is considered to be a robust model for insulin resistance and type 2 diabetes. Also, we observed that both pAMP and UA increased significantly in plasma from mice fed a high-fat diet compared with mice fed a standard diet (Fig. 1B). It is well known that the high levels of FFAs in the plasma were the main metabolic abnormality observed in obesity. Therefore, we investigated whether high FFAs could contribute to high pAMP. HUVECs were cultured and incubated with various FFA concentrations for different times. Cell injury was detected by fluorescence analysis of cells conjugated with PI. Incubation with FFA caused a dose- and time-dependent increase in endothelial cell injury (Fig. 1C), with maximum effects at 2 mM for 48 h. Extracellular 5'-AMP and UA, identified by HPLC, were elevated significantly in FFA-exposed groups compared with the control group (Fig. 1D).

Administration of 5'-AMP caused hyperglycemia and impaired insulin action in lean wild-type mice. Next, we investigated whether pAMP or UA was associated with the regulation of hyperglycemia. Synthesized 5'-AMP or UA was injected into wild-type mice to test the alteration of blood glucose levels. Glucose measurements demonstrated that 5'-AMP, not UA, caused hyperglycemia (Fig. 2A). The elevation of blood glucose was in a dose-dependent manner after 5'-AMP treatment (Fig. 2A). The 5'-AMP was biosynthesized by the dephosphorylation of adenosine 5'-triphosphate (ATP) released from intracellular stores and converted to adenosine by ecto-5'-nucleotides in the plasma. Therefore, we investigated whether the action of pAMP could be mediated by ATP or adenosine. Similarly to 5'-AMP, adenosine and ATP injection in mice induced hyperglycemia (Fig. 2C). Next, we investigated whether other nucleotides could also induce hyperglycemia. We injected mice with similar concentrations of inosine, CMP and IMP; blood glucose analysis showed that these nucleotides did not cause hyperglycemia (Fig. 2C), indicating that purine nucleotide signals were important in the regulation of blood glucose levels. Figure 2D showed that plasma levels of UA increased almost linearly with increasing doses of 5'-AMP, indicating the possibility that the high levels of UA in diabetic mice resulted from abnormal elevation of

Fig. 1. Plasma 5'-AMP (pAMP) and uric acid (UA) elevated in type 2 diabetic mice. A: HPLC analysis of 5'-AMP (pAMP) (left) and UA (right) in plasma of lean and \( db/db \) mice. Both the pAMP and plasma UA were elevated significantly in \( db/db \) mice. Each value is the mean ± SE of 5–7 animals. **P < 0.01 compared with lean mice. B: the pAMP (left) and plasma UA (right) were elevated significantly in high-fat (HF) diet-induced type 2 diabetic mice. Each value is the mean ± SE of 10 mice. **P < 0.01 compared with normal-feeding mice. C: dose and time course of free fatty acid (FFA) effects on cell injury in human umbilical vein endothelial cells (HUVECs) identified by propidium iodide. Representative images are shown. D: quantitation of extracellular 5'-AMP and UA in FFA-treating HUVEC culture. Each value is the mean ± SE; \( n = 3 \) independent experiments. *P < 0.05 and **P < 0.01 compared with control (CK) experiment.
pAMP. To identify whether injected 5’-AMP was beyond that in plasma of db/db mice, the dosage of 5’-AMP (0.5 μmol/g) was administrated in mice. HPLC analysis revealed an increase in pAMP level after 5’-AMP injection (Fig. 2E); the highest concentration of pAMP (~0.02 μmol/ml) was presented 30 min after 5’-AMP injection. The pAMP levels in plasma after 5’-AMP injection were similar to the pathological levels in db/db mice. Next, we investigated whether administration of 5’-AMP influenced insulin tolerance in fasted animals, which are used to evaluate the degree of insulin resistance. The results showed that 5’-AMP obviously decreased insulin sensitivity (Fig. 2F).

Injection with 5’-AMP had no influence in the insulin-binding affinity but attenuated insulin-dependent GLUT4 translocation. To explore how 5’-AMP influences insulin-signaling pathways, we investigated whether 5’-AMP could influence the binding affinity between insulin and its receptor. 125I-insulin binding to insulin receptors was carried out in red blood cells. After incubation in the presence or absence of various concentrations of 5’-AMP, specific 125I-binding was measured. Figure 3A showed that 5’-AMP had no influence on affinity of insulin binding to its receptor. We then analyzed whether pAMP influenced the subcellular localization of GLUT4 in skeletal muscle under fasting and maximally insulin-stimulated states. Insulin resistance in db/db mice is associated with decreased glucose uptake in insulin-sensitive tissue in skeletal muscle, in which glucose uptake is carried out by GLUT4 (20). The skeletal muscle distribution of the GLUT4 protein was visualized by immunofluorescence using a GLUT4-specific antibody coupled with a FITC-conjugated secondary antibody. Injection with 5’-AMP in vivo at 30 min resulted in a decline of the immunofluorescent signal that was detected readily in both the plasma membrane and intracellular vesicles (Fig. 3B), indicating that 5’-AMP attenuates insulin-dependent GLUT4 translocation in skeletal muscle. AMPK is the cellular mediator for many of the metabolic effects (31), and activation of AMPK stimulates GLUT4 translocation in skeletal muscle (9). The activation state of AMPK, as judged by phosphospecific antibodies to the activating T-loop phosphorylation site, was impaired significantly in the 5’-AMP-treated mice compared with control mice (Fig. 3, C and D). Moreover, mice injected with 5’-AMP displayed a marked decline in oxygen consumption (Fig. 3E).

The injection of 5’-AMP increased glucose-6-phosphatase activity and decreased glycogen level in livers. Glucose-6-phosphatase (G-6-Pase) is the critical hepatic enzyme that regulates glycogenolysis and glucogenesis (49). There was a significant positive correlation between hepatic G-6-Pase activity and blood glucose level, and elevated hepatic G-6-Pase activity was observed in db/db mice compared with wild-type mice (3). Therefore, we examined the effects of 5’-AMP on hepatic G-6-Pase activity in liver harvested 1 h after 5’-AMP injection. 5’-AMP injection resulted in a marked increase in hepatic G-6-Pase activity (Fig. 4A). We then performed a PTT
and measured glucose levels in mice treated with either 5'-AMP or saline. Pyruvate is used preferentially by the liver as a substrate for gluconeogenesis (48). As shown in Fig. 4B, PTT revealed a significant increase in glucose levels in mice treated with 5'-AMP compared with saline. Moreover, with 5'-AMP injection, hepatic glycogen levels declined to ~50% at 120 min (Fig. 4C).

**Elevation of pAMP increased intracellular adenosine levels.** A question that arose from the above observations was how pAMP induced a rapid and steep increase in blood glucose. It was well known that 5'-AMP could be dephosphorylated into adenosine by the membrane-bound extracellular enzyme in plasma. Then we investigated the role played by adenosine receptors in pAMP-induced hyperglycemia. Mice deficient in A1, A2a, A2b, or A3 adenosine receptors were compared with wild-type animals for their response to 5'-AMP. Both receptor-deficient and wild-type mice displayed no significant differences in their abilities to elevate blood glucose after administration of 5'-AMP (Fig. 5A). The findings suggested that pAMP-induced hyperglycemia was not directly related to adenosine receptor pathways. Then we investigated whether 5'-AMP injection could influence the levels of intracellular nucleotides. HPLC analysis showed that hepatic 5'-AMP and ADP did not obviously change after 5'-AMP injection, and the

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**Fig. 3.** Effects of 5'-AMP on insulin binding affinity and GLUT4 translocation. **A:** various concentrations of 5'-AMP had no effect on insulin binding affinity. **B:** immunofluorescence localization of muscle GLUT4 protein expression. Mice were fasted overnight and left untreated (left), given an intraperitoneal injection of insulin and glucose (middle), or given an injection of insulin, glucose, and 5'-AMP (right). **C:** Western blot analysis of the profile of AMPK and phosphorylated AMPK (p-AMPK) protein relative to actin in muscle of 5'-AMP-treated mice. Representative blots are shown. **D:** quantitation of AMPK and p-AMPK protein levels based on Western blotting profiles. **E:** oxygen consumption in wild-type mice with saline or 5'-AMP treatment. Mice with 5'-AMP showed significantly lower oxygen consumption than saline control. For **A** and **D,** each value is the mean ± SE of 5 animals. *P < 0.05 compared with control animals. For **B,** **C,** and **E,** results are presented as a representative figure from 3 independent experiments.

**Fig. 4.** Increased glucose-6-phosphatase (G-6-Pase) activity and decreased hepatic glycogen level after injection with 5'-AMP. **A:** enhanced hepatic G-6-Pase activities in mice injected with 5'-AMP. **B:** glucose appearance from pyruvate was increased in 5'-AMP-treated mice compared with saline control mice. **C:** hepatic glycogen level decreased following 5'-AMP injection. All samples were collected 1 h after injection of 5'-AMP (0.5 μmol/g) or saline, unless specifically indicated. Each value is the mean ± SE of 5–8 animals. *P < 0.05 and **P < 0.01 compared with control mice.
G6Pase mRNA was observed (Fig. 6A). Adenosine was present (Fig. 6D), implying that adenosine directly stimulates G-6-Pase activity.

Wild-type mice injected with 5'-AMP displayed phenotypes similar to db/db mice in glucose-related metabolic gene expression. In db/db mice, abnormal expressions of glucose-related metabolic genes were important characterizations in the development of hyperglycemia (2). Although G6Pase mRNA was not markedly different at the early stage of 5'-AMP injection, the mRNA expressions of fororkhead box O1 (Foxo1), including its regulated genes G6Pase and phosphoenolpyruvate carboxykinase (Pepeck), were markedly enhanced in livers of 5'-AMP-treated mice compared with control mice 1 h after 5'-AMP injection. Quantitative RT-PCR analysis showed that the mRNA levels of Glut2 were also elevated significantly in 5'-AMP-treated mice (Fig. 7A). The db/db mice displayed a similar expression pattern (Fig. 7B).

**DISCUSSION**

It was well known that FFAs contributed to the development of insulin resistance and type 2 diabetes. Plasma FFAs levels are elevated in most obese people, because FFA released from the enlarged and stressed adipose tissue is increased and FFA clearance is reduced (7). Acute elevations of plasma FFA inhibit insulin suppression of hepatic glucose production, and lowering of plasma FFA reduces chronic insulin resistance (44). Despite much work, the mechanism underlying FFA-induced insulin resistance is still not completely understood. Our results indicated that FFA-caused vein endothelial cell damages elevated pAMP levels in db/db mice and high-fat diet-induced diabetic mice; mice treated with 5'-AMP-induced hyperglycemia and displayed patterns similar to db/db mice in several glucose metabolic gene expressions and physiological responses. Also, 5'-AMP injection resulted in elevated plasma level of ATP obviously increased in the liver after 5'-AMP injection (Fig. 5B). Mice injected with 5'-AMP also decreased the ratio of 5'-AMP/ATP and ADP/ATP in skeletal muscle and adipose tissue (data not show). In db/db liver, a rise in ATP level was detected as observed in 5'-AMP-injected mice (Fig. 5C). Interestingly, whereas intracellular 5'-AMP failed to increase following 5'-AMP injection, we found the levels of intracellular adenosine to be increased four- to fivefold in livers 1 h after 5'-AMP injection compared with saline control (Fig. 5D), and we also observed a hyperadenosine liver in db/db mice (Fig. 5E).

Adenosine directly stimulated hepatic G-6-Pase activity. To clarify whether 5'-AMP-induced hyperadenosine could contribute to an increase in blood glucose, we first investigated the rate of adenosine elevation after 5'-AMP injection. Fifteen minutes after the injection of 5'-AMP, liver adenosine was increased threefold (Fig. 6A) and the hepatic G-6-Pase activity was significantly higher compared with saline control (Fig. 6B), whereas no significant difference in G6Pase mRNA was observed (Fig. 6C). Moreover, we determined that G-6-Pase activity in microsomal preparation was obtained in the the liver homogenates incubated with various concentrations of adenosine, and a significant increase in G-6-Pase activity was observed when adenosine was present (Fig. 6D), implying that adenosine directly stimulates G-6-Pase activity.

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levels of UA in a dose-dependent manner. Hyperuricemia was an independent predictor and an early biochemical marker of type 2 diabetes (12, 35). Thus, hyperuricemia in db/db mice could be associated with high levels of pAMP. The hyperuricemia was also observed in animal models that were fed high-fat diets (32). We suggested that altered levels of pAMP led to changes in UA content in type 2 diabetes. It was known that cell damage was experienced with high-fat diets prior to the development of hyperglycemia (13, 19), and hyperglycemia caused more tissue and even organ damage. Cell damage inevitably caused intracellular nucleotides to be released into the circulation (41). Thus, cell damage, especially FFA-induced vein endothelial cell damage, could be the source of pAMP in type 2 diabetes with abnormally high levels.

Whereas pAMP-induced hyperuricemia was independent of adenosine receptor pathways, we found cellular adenosine, but not 5'-AMP, to be markedly elevated in tissues after 5'-AMP treatment. Injection of 5'-AMP led to a rapid and steep increase (15 min after injection) in blood glucose that was independent of the increase in G-6-Pase mRNA. Our present data showed that adenosine directly stimulated G-6-Pase enzyme activity in a dose-dependent manner. G-6-Pase is a key limited enzyme in glucose homeostasis, which catalyzes the last biochemical reaction of glucose synthesis and the hydrolysis of G6P to glucose (49). Overexpression of G-6-Pase increases glucose production and causes hyperglycemia and glucose intolerance (52). Most previous studies have suggested that G-6-Pase activity is regulated primarily at the level of transcription (24, 47, 51). In keeping with a rapid effect of 5'-AMP on blood glucose under the fact of increased cellular adenosine, it has been strongly suggested that 5'-AMP-induced hyperglycemia was initiated by adenosine-stimulated G-6-Pase activity.

On the other hand, we observed that 5'-AMP injection resulted in impaired GLUT4 translocation in skeletal muscle. GLUT4 is expressed exclusively in insulin-responsive tissues such as skeletal muscle (23), in which insulin stimulates cellular glucose uptake by inducing the translocation of GLUT4 from an intracellular pool to the plasma membranes (10, 50). Because they are involved in the first step of the glucose utilization cascade, GLUT4 proteins are highly regulated in physiological as well as pathophysiological states. Impaired GLUT4 translocation has been shown to be linked to reduced glucose utilization in muscle of insulin-resistant and type 2 diabetic subjects (15, 40, 59). The insulin-stimulated translocation of GLUT4 was reduced significantly in both skeletal muscle and adipose tissue of diabetic mice (16, 30). Therefore, decreased muscle GLUT4 translocation was one of the potential mechanisms that mediated the acute effects of 5'-AMP on elevation of blood glucose.

Moreover, 5'-AMP injection decreased levels of phospho-AMPK, an active form of AMPK. Phosphorylation of AMPK results in enhanced fatty acid oxidation and decreased production of glucose (28). Recent studies have shown that AMPK is the cellular mediator for many of the metabolic effects of drugs such as metformin and thiazolidinediones (42). That chemical activation of AMPK in vivo with 5-aminoimidazole-4-carboxamide ribonucleoside improved blood glucose concentrations in diabetic db/db mice (17). It must be noted that extracellular 5'-AMP (including pAMP) was not equal with intracellular 5'-AMP. In our observation, injection with 5'-AMP failed to increase levels of intracellular 5'-AMP but resulted in a decline of the ratio of 5'-AMP/ATP. A previous investigation demonstrated elevation of 5'-AMP/ATP-inhibited dephosphorylation as well as the promotion of phosphorylation of the AMPK (57). Thus, a decrease in cellular ratio of 5'-AMP/ATP caused a reduction in pAMP, resulting in impaired activities of AMPK and decreased glucose utilization.

In the present study, we also demonstrated that mice treated with 5'-AMP mimicked db/db mice in the expression of several glucose metabolic genes and in physiological responses. The relationship between pAMP and phenotypes in db/db mice was supported by several findings: 1) pAMP caused hyperglycemia; 2) pAMP resulted in a decrease in insulin sensitivity; 3) pAMP enhanced gene expression of Glut2, Foxo1, Pepck, and G6Pase in livers; and 4) pAMP decreased metabolic rate. All of these phenotypes were also observed in db/db diabetic mice (2, 4, 14, 33).

Type 2 diabetes is a chronic and complex metabolic disease involving progressive development of insulin resistance and hyperglycemia. Our results and conclusions were derived from an acute model, and the role of elevated pAMP in a chronic model needs to be investigated further. It is quite possible that type 2 diabetes is triggered by FFA-induced vein endothelial cell damage, resulting in a rise in pAMP and, subsequently, excessive glucose production and insulin resistance. Compensatory hyperinsulinemia helps maintain normal glucose levels. Once the β-cells of the pancreas are unable to overcome insulin resistance, glucose levels rise, and a diagnosis of type 2 diabetes can be made. Hyperglycemia induces further damage in multiple organs, thus releasing more pAMP and causing more severe insulin resistance. Eventually, a cluster of abnormalities in multiple organs leads to metabolic syndrome.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS

Y. Zhang, Z.W., Y. Zhao, and M.Z. performed the experiments; Y. Zhang, Z.W., Y. Zhao, S.W., and J.Z. analyzed the data; Y. Zhang and J.Z. prepared the figures; Z.H. and J.Z. drafted the manuscript; Z.H. and J.Z. edited and revised the manuscript; J.Z. did the conception and design of the research; J.Z. interpreted the results of the experiments; J.Z. approved the final version of the manuscript.

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